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A expressão diferencial de genes WRKY de soja [*Glycine max* (L.) Merrill] em resposta à seca e a caracterização de seus promotores

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Dissertação submetida ao Programa de Pós-graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do titulo de mestre em Genética e Biologia Molecular

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RESUMO

A expressão diferencial de genes WRKY de soja [*Glycine max* (L.) Merrill] em resposta à seca e a caracterização de seus promotores

A soja é uma cultura de grande importância na economia mundial. No Brasil, o complexo da soja (grão, farelo e óleo) é responsável por uma parcela significativa das exportações. No entanto, o sucesso da produção é dependente de muitos fatores bióticos e abióticos. A ocorrência de déficit hídrico talvez seja hoje, e mais ainda no futuro, o principal desafio das culturas produtoras de grãos. Sendo assim, a obtenção de cultivares de soja tolerantes à desidratação é de grande interesse. Por ser de caráter poligênico, a tolerância à seca é difícil de ser trabalhada no melhoramento genético clássico. Sendo assim, o uso da engenharia genética apresenta-se como caminho para alcançar este atributo. A regulação transcricional de genes endógenos e a busca da precisão no controle de transgenes são as bases das estratégias de engenharia genética, que apostam nos fatores de transcrição e nos promotores induzíveis para desenvolver plantas tolerantes a estresses abióticos. As proteínas WRKY formam uma grande família de fatores de transcrição que está envolvida em processos fisiológicos e bioquímicos importantes nos vegetais, entre eles, a resposta das plantas ao déficit hídrico. A determinação do perfil de expressão de genes da família WRKY em condição de seca e a avaliação da atividade de seus promotores são os objetivos deste trabalho. Dez genes WRKY induzidos em situação de seca foram selecionados para o estudo: Glyma15g11680, Glyma09g37470, Glyma01g31920, Glyma07g02630, Glyma08g23380, Glyma05g36970, Glyma05g20710, Glyma05g29310, Glyma09g41050, Glyma08g15050. A análise *in silico* da sequência promotora desses genes revelou a presença de *cis*-elementos relacionados à resposta da planta a estresses. O perfil de expressão determinado por RT-qPCR mostrou que os genes Glyma15g11680, Glyma07g02630, Glyma05g36970, Glyma08g15050 e Glyma08g23380 são expressos diferencialmente entre os genótipos tolerante e suscetível e também entre folhas e raízes. Para fins de caracterização funcional dos promotores, fragmentos de 500bp e 2000bp a

montante do sítio de início da transcrição dos genes Glyma05g36970, Glyma08g15050 e Glyma15g11680 foram clonados em vetores apropriados para transformação genética de plantas. Foram obtidas plantas de tabaco transformadas pelo sistema *Agrobacterium tumefaciens*.

ABSTRACT

The differential expression of soybean [*Glycine max* (L.) Merrill] WRKY genes in response to drought and characterization of their promoters

Soybean is a crop of great importance for the world economy. In Brazil, the soybean complex (beans, meal and oil) is responsible for a significant portion of exports. However, the successful production is influenced by biotic and abiotic factors. The occurrence of drought is today, and perhaps even more in the future, the main challenge of grain crops. Thus it is of great interest to obtain soybean cultivars tolerant to desiccation. The drought tolerance trait is difficult to obtain through classical breeding due to its polygenic basis. In this context, genetic engineering is presented as a way to achieve this attribute. The transcriptional regulation of endogenous genes and the precise control of transgenes are the foundations of genetic engineering strategies that use transcription factors and inducible promoters to develop plants tolerant to abiotic stresses. The WRKY proteins form a large family of transcription factors that are involved in important physiological and biochemical processes in plants, including the response to water deficit. The goals of this work are the determination of the expression profile of WRKY genes under drought conditions and the evaluation of its promoters activity. Ten WRKY genes which are induced by drought were selected for this study: Glyma15g11680, Glyma09g37470, Glyma01g31920, Glyma07g02630, Glyma08g23380, Glyma05g36970, Glyma05g20710, Glyma05g29310, Glyma09g41050 and Glyma08g15050. The in silico analysis of these gene promoter sequences revealed the presence of cis - elements related to plant stress responses. The expression pattern determined by RT- qPCR showed that Glyma15g11680, Glyma07g02630, Glyma05g36970, Glyma08g15050 and Glyma08g23380 genes are differentially expressed between tolerant and susceptible genotypes and between leaves and roots. For the functional characterization of the promoters, 500 and 2000 bp fragments upstream of the transcription start site of Glyma05g36970, Glyma08g15050 and Glyma15g11680 genes were cloned

into appropriate vectors for plant genetic transformation. Tobacco plants transformed by *Agrobacterium tumefaciens* system were obtained.

LISTA DE ABREVIATURAS E SIGLAS

ABA: *abscisic acid*, ácido abscísico

ABRE: ABA responsive element, elemento responsivo ao ABA

AmpR : resistência à ampicilina

bp: *base pair*, pares de bases

CaMV: *cauliflower mosaic virus*, vírus do mosaico da couve-flor

cDNA: *complementary DNA*, DNA complementar

CO_2: gás carbônico

CONAB: Companhia Nacional de Abastecimento

DNA: ácido desoxirribonucleico

dNTP: desoxirribonucleos trifosfatados

EMBRAPA: Empresa Brasileira de Pesquisa Agropecuária

Gm: *Glycine max*

Glyma: *Glycine max*

GUS: β-glicuronidase

gusA: gene repórter que codifica a β-glicuronidase

KanR: resistência à canamicina

kbp: quilopares de bases

LB: *left border*, borda esquerda do T-DNA

LEA: *Late embryogenesis abundant protein family,* família de proteínas abundantes na embriogênese tardia

MAPA: Ministério da Agricultura, Pecuária e Abastecimento

min: minuto(s)

mM: milimolar(es)

MYB: *Myeloblastosis Oncogene protein family*, família de proteínas oncogênicas isoladas do vírus da mieloblastose

MYBR: *MYB recognition sequence, s*equência de reconhecimento das proteínas MYB

MYC: *Myelocytomatosis Oncogene proteins family*, família de proteínas oncogênicas isoladas do vírus da mielocitomatose

MYCR: *MYC recognition sequence, s*equência de reconhecimento das proteínas MYC

PCR: *polymerase chain reaction*, reação em cadeia da polimerase

PEG: polietilenoglicol

PLANTPan: *Plant Promoter Analysis Navigator*

RB: *right border*, borda direita do T-DNA

RNA: ácido ribonucleico

RT-qPCR: *Reverse Transcription quantitative PCR*

s: segundo(s)

Sm/SpR: resistência à estreptomicina e à espectinomicina

SAGE: *Serial analysis of gene expression,* Análise Serial da Expressão Gênica

T-DNA: transfer DNA, DNA de transferência

TFs: transcription factors, fatores de transcrição

T_0, T_{25} ,T_{50} ,T_{75} ,T_{100} , T_{125} ,T_{150} : tempo de exposição ao déficit hídrico em minutos

U: u*nit(s)*, unidade(s)

WT: *Wild-type,* selvagem

µl: microlitro(s)

SUMÁRIO

INTRODUÇÃO GERAL

1 INTRODUÇÃO GERAL

1.1 A SOJA E O DÉFICT HÍDRICO

A soja é a leguminosa cultivada mais importante do mundo (Clemente e Cahoon, 2009). Sua biomassa é explorada para fins alimentícios (farinhas e rações), químicos (resinas, tintas e solventes) e energéticos (biodiesel) (MAPA 2008). Tamanha versatilidade garante a esta cultura grande expressividade no cenário econômico. No Brasil, segundo maior produtor mundial, o complexo de soja (grão, farelo e óleo) está entre as três maiores exportações, totalizando 1,16% do PIB brasileiro em 2011 (CONAB 2013).

A restrição hídrica é o principal fator limitante do rendimento da soja. Nesta espécie, os períodos de maior suscetibilidade à restrição hídrica acontecem entre a germinação das sementes e a emergência de plântulas e entre a floração e o enchimento de grãos. A ocorrência de déficit hídrico nestas ocasiões é potencialmente prejudicial para o desempenho da cultura no campo, podendo diminuir a germinação das sementes, causar aborto das flores e reduzir o peso dos grãos (Doss e Thurlow 1974; Câmara e Heiffig 2000).

Apesar das condições edafoclimáticas brasileiras serem consideradas favoráveis para a produção de soja (MAPA 2008), episódios de secas prolongadas durante o verão tem se tornado cada vez mais comuns nos últimos anos, causando sérios prejuízos aos produtores (Brando e col. 2010). O rendimento das últimas safras reflete muito bem a relação de dependência entre a disponibilidade de água e o sucesso da produção. No verão do ano 2012, uma severa e longa estiagem motivada pelo fenômeno La Niña provocou a quebra das lavouras de grãos. Naquele ano, 66,68 milhões de toneladas de soja foram colhidas no país, 8,7 milhões a menos do que na safra anterior (CONAB 2012). No verão seguinte, o fenômeno El Niño causou o aumento e a melhor distribuição das chuvas que, adicionados à ampliação das áreas de cultivo, garantiram uma produção nacional recorde de soja, totalizando 81.456,7 milhões de toneladas colhidas (CONAB 2013b).

A água é essencial para a vida das plantas, compreende cerca de 90% da massa dos tecidos vegetais em crescimento (Farias e col. 2007). Cumpre

funções cruciais para o bom desenvolvimento e equilíbrio metabólico dos vegetais como termorregulação, transporte e manutenção da estabilidade de moléculas, expansão celular, filtragem de resíduos e promoção do suporte mecânico (Nepomuceno e col. 1994; Wehner e col. 2003; Zonia e Munnik 2007; Taiz e Zeiger 2009). Devido ao protagonismo da água em quase todos os processos fisiológicos e bioquímicos, o seu provimento insuficiente demanda o trabalho de um complexo de mecanismos que, em conjunto ou isoladamente, promovem a tolerância ao estresse (Casagrande e col. 2001).

A definição da resposta fisiológica específica ao déficit hídrico é na verdade estabelecida a partir das combinações de eventos moleculares que são ativados ou desativados pela percepção do estresse (Bray 1993). Os genes induzidos em condições de seca podem ser divididos em dois grupos: os codificantes de proteínas funcionais e os codificantes de proteínas regulatórias. O primeiro grupo está diretamente envolvido na tolerância ao estresse e adaptação celular. As proteínas de canais de água que aumentam a permeabilidade da membrana celular, as enzimas necessárias para a síntese de osmoprotetores (açúcares e betaínas), as proteínas protetoras de macromoléculas e membranas (proteínas LEA, osmotina, proteínas anticongelantes, chaperonas), as proteases (Thiol, Clp, e ubiquitinas) e as enzimas de detoxificação (glutationa-S-transferase, epóxido hidrolase solúvel, catalase, superóxido dismutase e ascorbato peroxidase) são exemplos de produtos da expressão deste grupo. Já os produtos do segundo grupo atuam como sinalizadores do estresse hídrico e reguladores da expressão dos genes que compõem o primeiro grupo (Shinozaki e Yamaguchi-Shinozaki 1997).

Por ser uma característica poligênica e consequentemente difícil de ser trabalhada no melhoramento genético clássico, a tolerância à seca muitas vezes não recebe o merecido destaque nos programas de melhoramento de plantas (Beever 2000). Logo, o número de cultivares tolerantes à carência hídrica ainda é pequeno. A fim de colaborar para o preenchimento desta lacuna, a biologia molecular assume um papel fundamental na identificação de genes envolvidos nas respostas ao déficit hídrico, aumentando o entendimento das rotas metabólicas envolvidas nas respostas fisiológicas à seca e, desta

forma, revelando caminhos mais eficazes para o desenvolvimento de plantas produtivas sob condições de estresse hídrico (Casagrande e col. 2001).

1.2 FATORES DE TRANSCRIÇÃO - PROTEÍNAS WRKY

Adaptar-se à variação das condições ambientais requer um elevado grau de plasticidade que é principalmente determinado pelo genoma das plantas. Os vegetais têm a capacidade de reprogramar o transcriptoma de uma maneira altamente dinâmica e temporal (Wang e col. 2009; Swanson-Wagner e col. 2012). Essa reprogramação que possibilita às plantas não somente a sobrevivência, como também a reprodução em ambientes desfavoráveis, ocorre principalmente pela atuação de uma rede de fatores de transcrição.

Os fatores de transcrição são proteínas que modificam a expressão de seus genes-alvo pela ligação a *cis*-elementos, pequenas sequências conservadas de DNA que compõem a porção promotora dos genes e têm função reguladora. Eles modificam a taxa de transcrição de seus genes-alvo através de interação (direta ou indireta) com a maquinaria transcricional basal (Guarente and Bermingharn-McDonogh 1992). Dos 46430 genes codificantes de proteína preditos no genoma da soja, 5671 foram classificados como supostos codificantes de fatores de transcrição, correspondendo a uma significativa parcela de 12.2% dos genes (Schmutz e col. 2010).

O papel regulatório dos fatores de transcrição e a interação com diversas proteínas faz com que o efeito da atuação de somente um deles atinja proporções genômicas, podendo refletir diretamente no fenótipo da planta (Riechmann e Ratcliffe 2000). A possibilidade de modular a expressão de muitos genes com a introdução de apenas um, colocou os fatores de transcrição na posição de ferramentas biotecnológicas ideais para o desenvolvimento de cultivares tolerantes a estresses. A defesa da planta aos estresses abióticos é concebida por um grande elenco de genes. Sendo assim, o emprego de genes codificadores de fatores de transcrição na engenharia genética facilitaria muito o cumprimento da complicada missão dos melhoristas

de sincronizar a expressão de vários genes para que a planta atinja o perfil tolerante.

Os fatores de transcrição são grandes regentes da sinalização em resposta ao estresse hídrico, exercendo inclusive a função de centros de conexão entre as rotas que regulam a reação da planta a esse estresse. As principais famílias de fatores de transcrição nesta rede são MYB, bHLH, bZIP, ERF, NAC, e WRKY (Tripathi e col. 2013).

As proteínas WRKY são assim chamadas, pois possuem em comum a sequência de aminoácidos WRKYGQK (ou WRKYGKK) . Essa sequência faz parte do domínio de ligação da proteína ao DNA, composto por 60 aminoácidos, que é seguido por uma estrutura do tipo “dedo-de-zinco” (Rushton e col. 2010). Em Eulgem e col. (2000), a família WRKY de fatores de transcrição foi dividida em três grupos baseando-se no número de domínios e nas particularidades da estrutura tipo “dedo-de-zinco”. As proteínas do grupo I possuem dois domínios WRKY, enquanto que as do grupo II contêm apenas um e são subdivididas em cinco subgrupos (do IIa ao IIe) de acordo com os motivos estruturais adjacentes ao domínio. Já o grupo III distingue-se por apresentar um motivo dedo-de-zinco C_2HC diferente do padrão C_2H_2 característico dos demais. A partir de uma análise mais detalhada e baseada também na filogenia, Zhang e Wang (2005) uniram alguns dos subgrupos II e reorganizaram os WRKY em cinco grupos I, IIa+b, IIc, IId+e e III. Entretanto, no estudo específico sobre as proteínas WRKY em soja, Yin e col. (2013) consideraram os resultados das suas análises mais consistentes com a primeira forma de agrupamento, retomando então a classificação sugerida por Eulgem e col. (2000) para descrever os componentes da família WRKY em soja.

Ao formarem complexos proteicos e se ligarem ao cis-elemento W-Box (C/TTGACT/C) presente nos promotores dos seus genes- alvo, os fatores de transcrição WRKY conduzem a expressão dos mesmos em processos fisiológicos e bioquímicos importantes nos vegetais, tais como as respostas a estresses bióticos e abióticos, senescência de folhas e metabolismo secundário

(Feng e col. 2012). Yin e col. (2013) anotaram 133 genes WRKY identificados no genoma da soja.

Conforme revisto por Tripathi e col. (2013), as proteínas WRKY cumprem um papel relevante na reação dos vegetais à deficiência de água. Dois genes WRKY de soja (*Glycine max L*), GmWRKY54 e GmWRKY20, analisados respectivamente por Zhou e col. (2008) e Luo X e col. (2013), conferiram tolerância à seca quando superexpressos em *Arabidopsis*. Ainda em Zhou e col. (2008) foram investigadas, através de RT-PCR (Reação em cadeia da polimerase precedida de transcriptase reversa) e posterior quantificação por densitometria, a expressão dos genes WRKY em plântulas de soja submetidas a estresses por déficit hídrico, salinidade e baixas temperaturas. O resultado foi que dos 64 genes avaliados, 24 foram induzidos em condições de escassez hídrica. Entre esses, somente dois apresentaram expressão diferencial unicamente sob ação do déficit hídrico, todos os demais genes foram induzidos por dois e até mesmo pelos três tipos de estresses avaliados. Corroborando estas observações, Yamaguchi-Shinozaki e Shinozaki (1999) demonstraram que plantas de *Arabidopsis* tolerantes a múltiplos estresses foram obtidas a partir da introdução de um único gene codificante de fator de transcrição.

1.3 PROMOTORES

Os promotores são regiões não codificantes do DNA que contêm informações cruciais para a ativação ou repressão de genes (Azad e col. 2011). Neles estão contidos os motivos de ligação para a RNA polimerase e complexos proteicos que, juntos, formam o maquinário capaz de definir a transcrição do gene em tempo, local e intensidade. Por este motivo, os promotores são considerados unidades de DNA fundamentais para regular a expressão gênica em nível da transcrição (Griffiths e col. 2002).

Em geral, considera-se como região promotora a fração de aproximadamente 1 a 4 kbp à montante do sítio de inicio da transcrição (Rombauts e col. 2003; Shahmuradov e col. 2005). No entanto, as formas

variadas nas quais um promotor pode se apresentar, inclusive à jusante do sítio de inicio da transcrição como um dos íntrons, dificulta a definição dos seus limites (Rombauts e col. 2003). Para identificar as zonas funcionais dentro de promotores, a estratégia de criar construções nas quais genes repórteres são fusionados a fragmentos resultantes de uma série de deleções da região promotora tem sido bastante usada (Chiera e col. 2007; Yan e col. 2014). A análise dos padrões de expressão dessas construções em plantas transgênicas serve como guia para o mapeamento das regiões reguladoras (Picot e col. 2010). Em seus trabalhos, She e col. (1993), Cho e col. (1995) e Strömvik e col. (1999), oferecem evidências de que 2 kbp da fração promotora dos genes da soja contém os motivos específicos necessários para ativação plena da expressão gênica.

A classificação dos promotores baseia-se no tipo e grau de controle que eles exercem sobre a expressão dos genes. Os promotores podem ser constitutivos, quando induzem os genes a expressarem-se de modo constante e ubíquo, ou induzíveis, quando respondem a sinais moleculares. Esses sinais atingem os promotores em forma de fatores de transcrição que se ligam a sítios específicos, denominados elementos reguladores *cis*-atuantes ou simplesmente *cis*-elementos. O tipo, o número, a posição e a combinação desses elementos promovem variações no padrão de transcrição dos genes, direcionando a expressão a um determinado tecido, estádio de desenvolvimento e/ou resposta a estresses ambientais (Zou e col. 2011).

Partindo do princípio de que a produção de organismos transgênicos de interesse comercial é baseada na elaboração de métodos capazes de controlar a expressão do transgene de maneira precisa, a busca por promotores induzíveis adequados para emprego em engenharia genética torna-se fundamental (Kuroki 2012). A resposta da planta a estresses abióticos é multigênica e complexa. Por isso, a obtenção de uma cultivar altamente produtiva, mesmo em condições ambientais desfavoráveis, exige um ajuste fino da expressão gênica que apenas é conquistado com o uso de promotores induzíveis específicos.

Para a transformação genética de plantas, dicotiledôneas em particular, os promotores comumente utilizados são o 35S do vírus do mosaico da couve flor (CaMV 35S) e os promotores dos genes da nopalina -sintetase e octopina -sintetase de *Agrobacterium tumefaciens,* todos de natureza constitutiva (Sassaki 2008). Embora muito importantes na conquista dos avanços em biologia molecular de plantas nas últimas décadas, a expressão dirigida por esses promotores está longe de ser a ideal para alcançar os resultados esperados. Ao manter a expressão do gene ligada durante toda a vida e em todos os tecidos da planta transformada, os promotores constitutivos não só geram um desperdício de energia como também afetam drasticamente as características agronômicas da planta (Caiyin e col. 2007). Liu e col. (1998), Yamaguchi-Shinozaki e Shinozaki (1999) e Chen e col. (2009) observaram um severo retardo no crescimento e redução da produção de sementes nas plantas modificadas geneticamente para expressar constitutivamente genes que codificam fatores de transcrição.

Mais do que o aprimoramento das técnicas de engenharia genética, na pesquisa de promotores vegetais também está depositada a expectativa de produzir plantas geneticamente modificadas que sejam mais bem aceitas pela sociedade. A substituição dos promotores constitutivos pelos induzíveis garantirá um controle mais efetivo da expressão dos transgenes, reduzindo, deste modo, os riscos de expressão indesejada, principalmente daqueles transgenes que conferem resistência a antibióticos e são usados para a seleção de transformantes (Huang e col. 2001). Além disso, a identificação e a caracterização de novos promotores gênicos vegetais aumentarão as opções de sequências promotoras disponíveis para uso em engenharia genética, diminuindo a dependência de empresas ou grupos de pesquisa detentores dos direitos intelectuais de sequências promotoras (Weber, 2011).

A soja é a cultura que tem a maior área cultivada de plantas transgênicas no mundo. Sendo assim, os esforços para obter avanços e lançar cultivares que superem as que já estão no mercado são constantes, aumentando ainda mais a necessidade de descoberta e caracterização de promotores nativos da espécie. Segundo Yin e col. (2013), o *cis*-elemento ABA responsivo (ABRE) é um dos mais abundantes nos promotores dos genes

WRKY da soja. Evidenciando o papel destacado do ácido abscísico (ABA) na regulação dos fatores de transcrição WRKY nesta espécie e sugerindo a atuação dessas proteínas em rotas de resposta a estresses abióticos, visto que a atuação do ABA como sinalizador deste tipo de estresse é conhecida. Em soja, já foram estudados promotores induzíveis por auxina (Li e col. 1994), por baixas temperaturas (Chen e col. 2009), pela infecção do nematóide do cisto da soja, além de promotores constitutivos (Chiera e col. 2007; Hernandez-Garcia e col. 2010) e semente-específicos (Cho e col. 1995; Buenrostro-Nava e col. 2006; Caiyin e col. 2007). Entretanto, devido ao grande impacto negativo causado pela seca na produção de soja, a pesquisa sobre os promotores induzíveis por déficit hídrico precisa ser aprofundada.

OBJETIVOS

2 OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo deste trabalho é a identificação de genes WRKY da soja que apresentem acentuada expressão em condições de déficit hídrico e a caracterização de seus promotores.

2.2 OBJETIVOS ESPECÍFICOS

1) Identificar dez genes da família WRKY superexpressos em condição de déficit hídrico;
2) Validar o perfil de expressão dos genes candidatos em plantas de duas cultivares de soja submetidas à restrição hídrica;
3) Caracterizar os promotores destes genes utilizando ferramentas de análise *in silico*;
4) Obter plantas de tabaco transgênicas que expressem o gene repórter *gusA* direcionado pelos promotores WRKY em estudo.

CAPÍTULO I

The differential expression of soybean WRKY genes in response to drought and characterization of their promoters

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Manuscrito em redação

Differential expression of soybean WRKY genes in response to drought and characterization of their promoters

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Key-words: *Glycine max*, drought tolerance, transcription factors, WRKY, promoters, genetic transformation

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) has a great importance for the world economy. Its grain has a high versatility that gives rise to products and subproducts widely used by agribusiness (production of oilseed and animal feed), chemical and food industry. Recently, the soybean use is also increasing as an alternative source of biofuel (Masuda and Goldsmith 2009).

Drought stress is a major constraint to soybean production, reducing yield by about 40 % (Specht et al. 1999). Therefore, engineering drought tolerance in plants has huge economical worth (Umezawa et al. 2006).The development of cultivars tolerant to dehydration is a challenging task for breeders. The goal of plant breeding is to accumulate genes that contribute to plant stress tolerance (Varshney et al. 2011). However a critical point is to

define which and how many genes are necessary to combine in one plant in order to make it tolerant to a stress response, which involves a large genetic network and complex molecular mechanisms. Given the difficulty in deciphering this puzzle emerges the strategy of using regulatory genes as objects of genetic engineering, requiring the introgression of only one or a few genes for development of drought tolerant genotypes. Among the various classes of regulatory genes, those encoding transcription factors are a wise choice to implement this strategy. This is because the acting of transcription factors in the recruitment / blocking RNA polymerases to DNA thereby activating/repressing gene transcription, makes them true orchestrators of plant response to environmental variations (Udvardi et al. 2007). The signal transduction pathways can ultimately regulate the expression of drought-responsive genes through transcription factors (Ku et al. 2010). In the soybean genome, about 500 transcription factors were *in silico* annotated (Mochida et al. 2009), among them are the WRKY proteins.

The WRKY proteins, named because of the invariant WRKY amino acid sequence at the N-terminus, are a group of zinc-finger proteins with a Cx4–5Cx22–23HxH or Cx7Cx23HxC structure (Eulgem et al. 2000). The WRKY transcription factors comprise one of the largest families of regulatory proteins in plants (Eulgem and Somssich 2007). Besides many developmental processes such as seed germination, flower development, secondary metabolism, morphogenesis, dormancy and senescence, WRKY genes are involved in regulating the plant response to stress (Journot-Catalino et al. 2006; Li et al. 2006; Wang et al. 2006; Zheng et al. 2006; Feng et al. 2012). WRKY participation in modulating the plant response to biotic stresses has been well studied, but the construction of understanding the functioning of these proteins in abiotic stress has just started. Over the past six years studies in several plant species have reported the WRKY TFs differential expression and functioning under drought stress conditions (Ramamoorthy et al. 2008; Berri et al. 2009; Wu et al. 2009; Qiu and Yu 2009; Wang et al. 2009; Ren et al. 2010; Xiong et al. 2010; Shekhawat et al. 2011; Jiang et al. 2012; Niu et al. 2012; Luo et al. 2013; Wang et al. 2013), but few studies have been performed in soybean (Zhou et al. 2008)

In order to achieve the goals of genetic engineering, promoters that are able to regulate gene expression in a highly specific manner are demanded. The overexpression of transcription factor genes directed by the constitutive promoter CaMV35S, commonly used in plant transformation, does not reflect the tight and fine tuning transcription regulation of plant response to drought. It may cause severe growth retardation and reduction in seed production that can occur even under normal environmental conditions in transgenic crops with the constitutive expression of TFs (Liu et al. 1998). Because of that, the study of inducible promoters becomes a priority in the search for appropriate expression patterns to improve the transgenic work and the knowledge about gene regulation routes (Liu et al. 2013). In this paper, the promoter expression of Glyma05g36970, Glyma08g15050 and Glyma15g11680 genes was analyzed by heterologous transformation in tobacco plants and the transcription profiles of these and seven more WRKY genes were determined in soybean under water stress conditions.

MATERIAL AND METHODS

Selection of WRKY genes

Ten WRKY genes induced under drought conditions were selected based on SuperSage data, the serial analysis of gene expression, from the Soybean Genome Project database - Laboratório de Genômica e Expressão – UNICAMP (http://bioinfo03.ibi.unicamp.br/soja/). The access number of the select genes are: Glyma01g31920, Glyma05g20710, Glyma05g29310, Glyma05g36970, Glyma07g02630, Glyma08g15050, Glyma08g23380, Glyma09g37470, Glyma09g41050 e Glyma15g11680 (http://www.phytozome.net/).

Plant material

In an experiment conducted at Embrapa Soja, Londrina, Paraná, Brazil, the BR16 and EMBRAPA48 soybean cultivar plants, highly and slightly

sensitive to dehydration stress, respectively (Oya et al. 2004) were cultivated hydroponically in a greenhouse at 25 ° C ± 2 ° C and 60% ± 5% relative humidity, as described by Kulcheski et al. (2011). Seedlings in V2 stage were removed from hydroponic solution and kept in a tray in darkness and without nutrients. Leaves and roots were collected at 0 (control), 25, 50, 75, 100, 125, and 150 min after the initiation of dehydration stress and were frozen in liquid nitrogen and stored at −80 °C until RNA extraction as described by Martins et al. (2008). Three biological replicates (three plants/replicate) were sampled for each organ/genotype/treatment point. The RNA extraction from each sample and the cDNA synthesis reaction was performed according to the procedure described by Cabreira et al (2013). To evaluate the relative gene expression, the first-strand cDNA reaction product was diluted 1:100.

RT-qPCR

The gene expression was analyzed using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Primers (Table 1) were designed using Primer3 software (http://frodo.wi.mit.edu/) according to the genes sequences and the applied annealing temperature was 60 ° C. The reactions were performed in 25 µl volume containing 0,1 µM of each primer, 12.5 µl of diluted cDNA sample, 1× PCR buffer (Invitrogen), 3mM $MgCl_2$, 0,1mM of each dNTP, 4 µl SYBR-Green solution (1:100,000, Molecular Probes Inc., Eugene, OR) and 0.25 U Platinum Taq DNA Polymerase (Invitrogen). Real-time PCR was carried out on StepOne Applied Biosystem Real-Time Cycler in a 96-well reaction The PCR-cycling conditions were implemented as follows: 5 min at 94°C for an initial denaturation, 40 cycles of a 10-s denaturation step at 94 °C, a 15-s annealing step at 60 °C, and a 15-s extension step at 72 °C ending with 2 min at 72°C for a final extension. Technical quadruplicate reactions were performed for each sample. The f-box, ACTII, and ELF housekeeping genes (Jian et al. 2008) were used for normalization, following the methodology employed in Cabreira et al. (2013). The expression was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

Through the variance analysis statistical test, relative expression comparisons among dehydration stress time and between genotypes were performed for each organ separately. Comparison between organs (leaves x roots) was performed for each genotype. When necessary, data were transformed using the weighted least square method. Means were compared using the Bonferroni multiple comparison test. The SAS 9.2 and the SPSS/PASWSTAT 18 software packages were used to perform this analysis.

Promoter *in silico* analysis

The analysis of a 2,000-bp region upstream of the transcription start site of WRKY genes was performed using tools available at Plant Plan database (Chang et al. 2008) (http://plantpan.mbc.nctu.edu.tw/index.php).

Vector construction

The promoter fragments of WRKY genes were amplified from soybean DNA (Bragg cultivar) by PCR under the following conditions: 94°C – 5 min for pre-denature, 30 cycles at 94°C – 1 min, 60°C – 1 min, 72°C – 2 min, and 72°C – 5 min for post-extension. A high fidelity DNA polymerase was used. The primer sequences are listed in table 2. Two cloning methods were employed:

- **Restriction Enzyme Cloning Method**

The 2 kbp promoter fragment upstream the transcription start site of Glyma08g15050 gene was cloned into pGEM-T-Easy vector (Promega, Wisconsin,USA) (Fig.1a). In order to check the construction, the pGEM:Glyma08g15050$_{prom}$ vector was sequenced on an ABI PRISM 3100 Genetic Analyzer automatic sequencer (Applied Biosystems). The pGEM:Glyma08g15050$_{prom}$ was digested with *SacII* and *NotI* restriction enzymes and the fragment containing the promoter was inserted into the same cloning sites of the pGUSXX vector (Pasquali et al.1994) (Fig. 1b). The cassette

composed of the gusA gene and Glyma08g15050 gene promoter was excised from the vector pGUSXX:Glyma08g15050$_{prom}$ by the cleavage reactions of SacI and KpnI restriction enzymes and subcloned into the same sites of the binary vector pCAMBIA2300 (Fig. 1c).

- **Gateway Cloning System Method**

The 0,5 kbp and 2 kbp promoter fragments upstream the transcription start site of Glyma05g36970 and Glyma15g11680 genes and the 0.5Kbp promoter fragment of Glyma08g15050 were introduced into the pENTR/D-TOPO cloning vector (Invitrogen, Carlsbad, California, USA) according to manufacturer protocol (Fig. 2a). The formed entry vectors were recombined with the destination vector pKGWFS7 (Ghent University, Kortrijk, Ghent, Belgium) (Fig. 2b) thus forming the expression vectors (Fig. 2c). To confirm the authenticity of the vectors constructed, the presence of both fragments WRKY promoters as well as the reporter gene was verified by PCR reactions (Fig. 3). The forward and reverse primers sequences used to amplify 228bp of gusA gene sequence were 5′-GGTATCAGCGCGAAGTCTTTA-3′ and 5′-GGATAGTCTGCCAGTTCAGTTC-3′, respectively (T_a = 62°C). The primers employed for amplification of WRKY promoter fragments are listed in table 2.

Tobacco transformation

The expression vectors (Fig.1d, 2c) were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation and used to transform tobacco (*Nicotiana tabacum* L. cv SR1) according to the leaf disc method (Horsch et al. 1985).

RESULTS

Five of ten analyzed WRKY genes exhibit differential expression between genotypes and organs under plant dehydrating conditions

To allow comparisons between genes, genotypes and organs, all expression data were normalized based on the lowest transcript level detected, enabling the analysis of the expression profiles of 10 genes WRKY in response to water deficit. The transcriptional profiles of Glyma08g15050, Glyma05g36970, Glyma08g23380, Glyma07g02630 and Gl yma15g11680 showed higher expression levels in EMBRAPA48, the tolerant cultivar, when compared to the BR16, the susceptible one, at least at one of the six time points under dehydration (Figure 5). In leaves, statistically significant ($p<0.05$) higher transcription levels were observed at, T150 for Glyma08g15050 (Figure 5b), T50 for Glyma05g36970 (Figure 5d), and at T75 and T150 for Glyma15g11680 (Figure 5j). In roots, this pattern was observed at T100 and T150 for Glyma08g15050 and Glyma08g23380 (Figure 5b and e) and at all periods of plant exposure to drought stress for Glyma07g02630 (Figure 5g).For all other genes analyzed, no significant differences were detected between the susceptible and the tolerant cultivar (Figures 5a, c, f, h and i). The statistically significant increase in the expression of Glyma08g15050, Glyma05g36970, Glyma08g23380, Glyma07g02630 and Gl yma15g11680 genes in cultivar EMBRAPA48 compared to BR16 indicates a robust performance of these genes in building a profile tolerant to drought.

A comparison of gene expression levels between organs (leaves x roots) within each cultivar was performed (Figure 6). Glyma08g15050, Glyma05g36970 and Glyma15g11680 genes presented some points with higher expression in leaves of both genotypes (EMBRAPA48 and BR16). In this organ, Glyma08g15050 showed significantly higher expression at T150 in the tolerant genotype and at T100 and T150 in the susceptible (Figure 6b). Glyma05g36970 expression was higher at T75 in BR16 and at T50 in EMBRAPA48 (Figure 6d). For Glyma15g11680, the expression was significantly higher at T75 and T150 in EMBRAPA48 (Figure 6j). On the other hand, higher expression level was observed in roots

for Glyma08g23380 and Glyma07g02630 genes. For Glyma08g23380, the expression was higher in roots of both cultivars from T25 to T75 (Figure 6e). Glyma07g02630 gene expression in roots of EMBRAPA48 plants surpassed the expression in leaves at all evaluated points (Figure 6g). For the remaining genes, significant differences of transcriptional levels between organs were also detected (Figure 6a, f, h and i). However, all of them were statistically deemed as equally expressed between susceptible and tolerant genotypes (Figure 5 a, f, h and i).

WRKY gene promoters are enriched for W box binding sites and *cis*-elements related to water deficit stress

The *in silico* analysis showed that the putative promoters of genes studied are composed of *cis* elements related to the regulation of several physiological processes such as plant response to phytohormones (ABA, gibberellic acid, salicylic acid, auxin), infection, deficiency of nutrients (phosphorus, sulfur), light stress, high temperature stress and salinity. *Cis*-acting elements associated with root nodulation and flowering were also found. As expected, cis-elements involved in plant response to water stress as well as the Wbox, the DNA binding site of WRKY transcription factors, are present in all investigated putative promoters (Table 3).

Tobacco transformation

In the first experiment the construction "2000bp Glyma08g15050 promoter: gusA" was used to transform tobacco leaf discs. Nine putative transgenic tobacco plants were obtained (Fig. 4a). The DNA of transformants was extracted according to the Doyle & Doyle (1990) protocol and subjected to PCR reactions to confirm the presence of promoter fragment (Fig. 4b) and gusA reporter gene (Fig. 4c). The banding pattern of seven plants (1,3,4,5,6,7 and 8) confirms the transgenic condition. A second experiment was performed using the five gateway constructions (pKGWFS7:Glyma05g36970$_{500bp\ prom}$, pKGWFS7:Glyma05g36970$_{2000bp\ prom}$, pKGWFS7: Glyma08g15050$_{500bp\ prom}$

pKGWFS7: Glyma15g11680$_{500bp\ prom}$, pKGWFS7: Glyma15g11680$_{2000bp\ prom.}$ The putative transformants are under selection. Transgenic plants obtained will be subjected to reporter gene expression tests for the analysis of WRKY promoter activity.

DISCUSSION

Five of ten analyzed WRKY genes exhibit differential expression between genotypes and organs under plant dehydrating conditions

The genetic engineering of key regulatory genes that govern a subset of stress-related genes appears to be one of the most promising strategies for enabling scientists to minimize the deleterious effects associated with drought in plants (Umezawa et al. 2006). However, the effectiveness of this strategy depends on understanding how the genes encoding components of the regulatory machinery induced in conditions of water stress are expressed and controlled in order that the choice of genes is well-founded. The statistically significant increase in the expression of Glyma05g36970, Glyma07g02630, Glyma08g15050, Glyma08g23380 and Glyma15g11680 genes in the cultivar EMBRAPA48 compared to BR16 indicates a robust performance of these genes in building a profile tolerant to drought. This result point them, among the genes here studied, as the most likely candidates to be used in genetic engineering strategies which have as goal the achievement of soybean cultivars tolerant to dehydration.

Genes differentially expressed in roots under drought conditions are objects of great interest to crop breeding. Evidence of this is the strategy of pyramiding of genes involved in the production of compatible solutes in roots. Resulting in boosting of osmotic adjustment and root growth and thereby increasing the plant's capacity to capture water from deeper soil layers (Sinclair et al. 2004; Munns. 2005; Takeda and Matsuoka. 2008; Varshney et al. 2011). From the root tissues are transmitted chemical signals that inform the plant on the soil water status (Schachtman and Goodger 2008). In dry conditions, these signals regulate stomatal behavior, leaf initiation, leaf expansion and other

developmental processes (Davies and Zhang 1991). The Glyma07g02630 and Glyma08g23380 genes stood out due to their higher expression in roots of tolerant cultivar plants. Their ortholog in *Arabidopsis*, WRKY40 (Yin et al. 2013), was presented as a likely negative regulator of plant ABA response (Chen et al. 2010). This assumption was supported by the interaction of WRKY40 with the promoters of ABA responsive genes, ABI4 and ABI5, repressing their expressions (Liu et al. 2012). In addition, an increase in root length was observed in *Arabidopsis* lines overexpressing WRKY40 (Chen et al.2010).

The stress caused by water deficiency reduces leaf area and impairs photosynthesis due to decreased CO_2 assimilation resulting from stomatal closure (Stolf et al. 2009). The higher expression of Glyma05g36970, Glyma08g15050 and Glyma15g11680 genes in leaves of the tolerant cultivar may indicate their participation in regulatory routes that lead to minimizing the water deficit damaging effects. The role of WRKY53, Glyma05g36970 orthologous gene in *Arabidopsis* (Yin et al. 2013), as a component of the leaf senescence-inducing signaling complex was demonstrated by several studies (Miao et al. 2004, 2007a, 2007b, 2008; 2013; Zentgraf et al. 2010). Concerning Glyma08g15050 orthologous gene in *Arabidopsis*, WRKY51 (Yin et al. 2013), it is required for defense against bacterium *Pseudomonas syringae*, as reported by Gao et al (2011).

Besides being a plant mechanism used to avoid water loss under water stress conditions, the stomatal closure is part of the plant innate immune response in restricting bacterial invasion (Melotto et al. 2006). In the same way, the decline in photosynthetic activity, besides being one of the main effects of drought, is among the factors initiators of the leaf senescence (Zentgraf et al. 2010). Regarding these evidences is not only common but also expected that the plant senescence, bacterial attack and drought response pathways are interconnected by the performances of transcription factors as convergence points.

WRKY gene promoters are enriched for W box binding sites and *cis*-elements related to water deficit stress

The *cis*-acting elements are conserved regions of gene promoters that act as transcription factor binding sites, being in this way, crucial pieces of this complicated gear system which constitutes gene regulation. In this way, the analysis of c*is*-acting elements is important to understand the regulatory systems in stress-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki 2005). The definition of the correspondence between conserved promoter motifs and the behavior of gene expression has provided advances in molecular stress (Chen et al. 2012).

The functional diversity of *cis*-acting elements revealed by *in silico* analysis of the studied putative promoters was not a surprise considering the WRKY family multifaceted expression profile. The presence of Wbox in all investigated promoter regions reveals the complex regulatory network established by the components of this transcription factor family that involves not just the interaction with other proteins but also the self-regulation reported previously (Eulgem et al. 1999; Cormack et al. 2002; Robatzek and Somssich, 2002 and Dong et al. 2003).

Taking in to account the known involvement of WRKY family in plant response to stress efforts have been made to understand the relationship between these transcription factors and the phytohormone ABA, referred as the "stress hormone" due to its massive accumulation in the plant under environmental stress conditions (Davies et al. 1991; Oliveira 2010). The participation of specific WRKY genes in ABA signaling pathways has been demonstrated (Jiang and Yu, 2009; Ren et al. 2010 and Shang et al. 2010). Under drought conditions ABA operates mainly in promoting stomatal closure, thus reducing water loss by transpiration (Campalans et al. 1999). There are at least four pathways of gene expression induction in response to drought, two of them are ABA-dependent and contain ABRE (ABA responsive element), MYBR (MYB recognition sequence) and MYCR (MYC recognition sequence) as key *cis*-acting elements (Shinozaki and Yamaguchi-Shinozaki. 1997).

The MYB and MYC proteins depend on ABA accumulation to be synthesized, thereby indicating that their activation and consequently the activation of genes induced by them occur at the late stages of the stress responses (Nakashima and Yamaguchi-Shinozaki. 2006; Shinozaki and Yamaguchi-Shinozaki. 2007). However, despite the fact that WRKY are described as genes of immediate and transient response to stress (Eulgem et al. 2000), the *in silico* analysis showed that the studied promoters are enriched of MYBR e MYCR *cis*-acting elements (Table 3), suggesting a major involvement of MYC and MYB transcription factors in the regulation of WRKY genes. The direct interaction between MYBR and MYB2-box-like elements have been reported in the promoters of osmotic, drought, and ABA-induced genes (Urao et al. 1993; Jung et al. 2008). In addition, it has been observed a significant over-representation of MYBR in the promoter regions of pathogen/salicylic acid-regulated and unregulated *Arabidopsis* WRKY genes, indicating that MYB proteins may play a role in regulating the basal and constitutive expression of WRKY genes (Dong et al. 2003). The MYB2-box-like elements were the second most common transcription factor binding site motif found in the *in silico* analysis of 3922 plant promoters (monocotyledons and dicotyledons) carried out by Liu et al (2013).

In the present study, the investigation of putative WRKY promoters did not reveal the presence of *cis*-elements related to ABA independent pathways of response to water deficit, corroborating the role of WRKY proteins as regulatory factors of ABA`s action in plants, as revised by Rushton et al (2012).

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Table 1 Primer set used in the RT-qPCR to determine the expression profile of WRKY soybean genes

Genes		Prímers (5´→ 3´)
Glyma01g31920	Forward	GGTGGAGGAAATATGGACAGA
	Reverse	TGTGATTACGACTTATGAAGGGAA
Glyma05g20710	Forward	GATGGCTGATATTCCACCAGA
	Reverse	AACCTACGAGGGAGAGCACA
Glyma05g29310	Forward	ATGGGCATGGAGAAAGTACG
	Reverse	TTACTCCTCCGACCACAACC
Glyma05g36970	Forward	CCCAAATGGATGGATCATGTA
	Reverse	GCAACAAAGCAAGTGCAGAG
Glyma07g02630	Forward	AGCTGCCCTGTCAAAAAGAA
	Reverse	TGTACCGCATCTCTCAGCAC
Glyma08g15050	Forward	GTCCAAGGATCGCGTTTAGA
	Reverse	GAAGAAAAGGGTGGAAAGGG
Glyma08g23380	Forward	TGGACAAAAGGTGACCAGAG
	Reverse	AGCACAATCATCCTCAGGCT
Glyma09g37470	Forward	GCCAGTGGAGGAAATATGGA
	Reverse	CCTCTTCCAGTTTCAGCCAC
Glyma09g41050	Forward	CCTGAATGCCAAATTCCCTA
	Reverse	CATACTTGACCCCATGTCCC
Glyma15g11680	Forward	ATGGATGCCAATGGAGAAAA
	Reverse	ACAACATATGAAGGCACGCA

Table 2 Primers used to isolate and amplify promoter fragments of WRKY genes

Genes		Prímers (5´→ 3´) 0.5 kb promoter fragment	Prímers (5´ → 3´) 2 kb promoter fragment
Glyma05g36970	Forward	TGGACCCTATCATTAACCAAT	TCGATGAAAGCAAGATATTC
	Reverse	TTTTTGTGTTGTTTATAATATAGTCAAAAGG	TTTTTGTGTTGTTTATAATATAGTCAAAAGG
Glyma08g15050	Forward	CCGCCACAAGTCCTCAAC	AAGTAAGAAAACAGAAGAAAAA
	Reverse	TGCTGATAACTAAATATGGTAGTAAATG	TGCTGATAACTAAATATGGTAGTAAATG
Glyma15g11680	Forward	TGTGTCATAAGTGAAATTAAATGGTC	GAAACATTTATGTTCTCTAAAAA
	Reverse	ACTTGGTCCACCTCGCAGT	ACTTGGTCCACCTCGCAGT

Table 3 Number of copies and biological function of putative *cis*-elements related to water stress that were identified in the WRKY gene promoters

CIS- ELEMENT	CONSENSUS SEQUENCE	TRANSCRIPTION FACTOR	FUNCTION	Glyma01g31920	Glyma05g20710	Glyma05g29310	Glyma05g36970	Glyma07g02630	Glyma08g15050	Glyma08g23380	Glyma09g37470	Glyma09g41050	Glyma15g11680	REFERENCES
MYBR	YAACKG	MYB2CONSENSUSAT	MYB recognition site found in the promoters of the dehydration-responsive gene rd22	2	1		1		4	1	1	2		Abe et al. (2002)
MYBR	WAACCA	MYB1AT	MYB recognition site found in the promoters of the dehydration-responsive gene rd22	4	4	4	2	2	3	2	2	2	1	Abe et al. (2002)
MYBR	CTAACCA	AtMYB2	MYB recognition site found in the promoters of the dehydration-responsive gene rd22	1										Abe et al. (1997)
MYBR	TAACTG	MYB2AT	Binding site for MYB plant protein (ATMYB2) involved in regulation of water stress responsive genes			2			2	1	2		1	Urao et al. (1993)
MYBR	CNGTTR	MYBCORE		5	2	3	3	3	7	4	7	3	2	Abe et al. (1997)
MYCR	CANNTG	MYCCONSENSUSAT	MYC recognition site found in the promoters of the dehydration-responsive gene rd22	3	2	7	6	5	8	5	8	6	6	Abe et al. (2002)
MYCR	CACATG	AtMYC2	MYC recognition site found in the promoters of the dehydration-responsive gene rd22	1				1		3		2		Abe et al. (1997)
MYCR	CATGTG	MYCATERD1	MYC recognition sequence; necessary for expression of erd1 in dehydrated Arabidopsis		1						1	3		Simpson et al. (2003)
ABRE	ACGTGKC	ABRE	Cis-elements found in the promoters of the ABA-regulated genes and recognized by bZIP proteins									1		Yamaguchi-Shinozaki and Shinozaki (1997)
ABRE	BACGTGKM	ABRE-like							2			1	1	Shinozaki and Yamaguchi-Shinozaki (2000)
WBOX	TTGAC	WBOXATNPR1	W-box found in the promoter of Arabidopsis NPR1 gene; recognized specifically by WRKY DNA binding proteins	5	4	3	8	4	6	3	5	5	7	Yu et al. (2001)

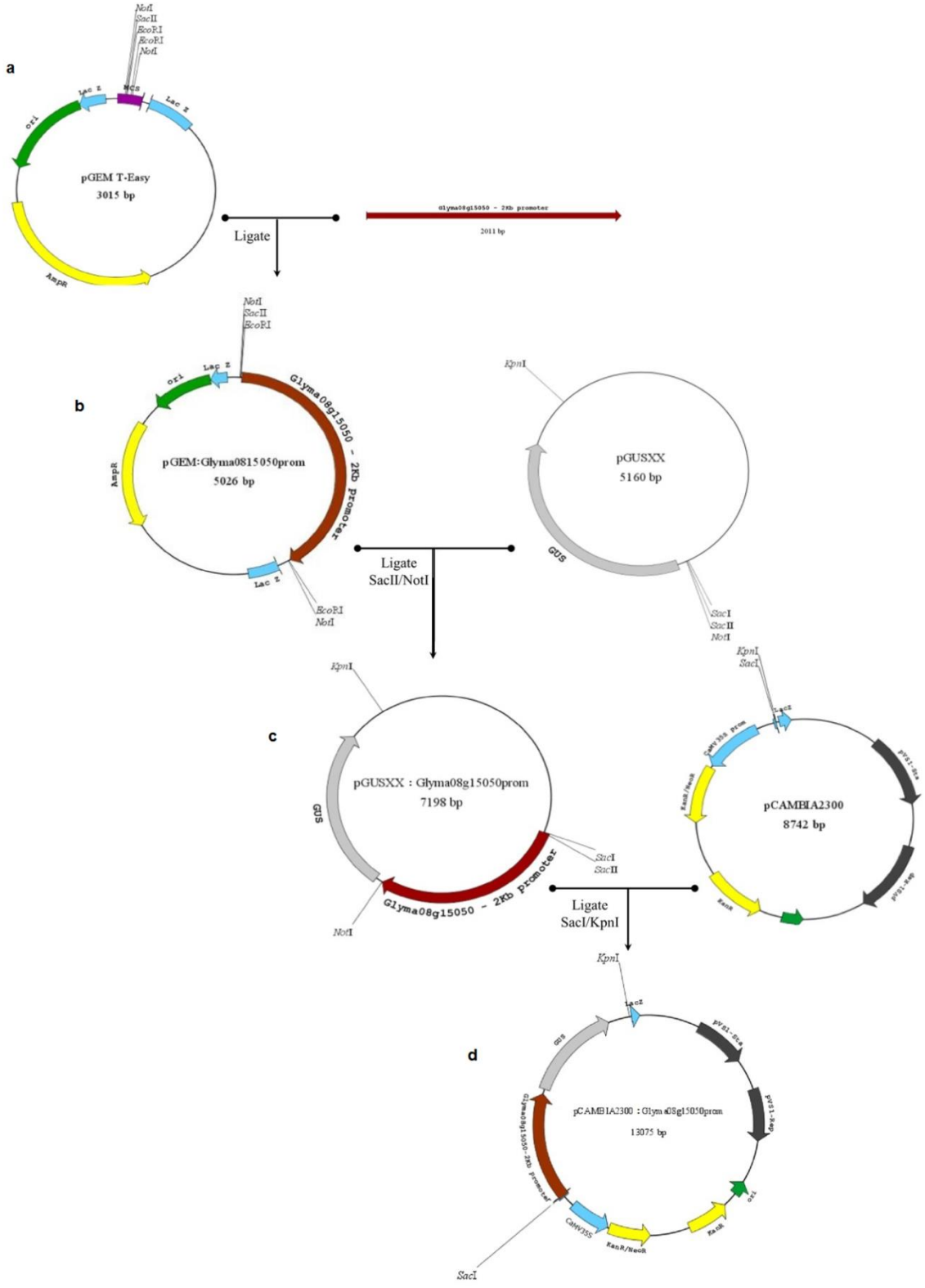

Fig.1 Construction of pCAMBIA2300:Glyma08g15050$_{prom}$ vector by Restriction Enzymes Cloning Method. (A) The 2 kbp promoter fragment upstream the translation start site of Glyma08g15050 gene was cloned into pGEM-T-Easy vector . (B) The pGEM:Glyma08g15050$_{prom}$ was digested with *SacII* and *NotI* restriction enzymes and the fragment containing the promoter was inserted into the same cloning sites of the pGUSXX vector. (C)The cassette composed of the gusA gene and Glyma08g15050 gene promoter was excised from the vector pGUSXX:Glyma08g15050$_{prom}$ by the cleavage reactions of *SacI* and *KpnI* restriction enzymes and subcloned into the same sites of the binary vector pCAMBIA2300, (D) resulting in the pCAMBIA2300:Glyma08g15050$_{prom}$ expression vector.

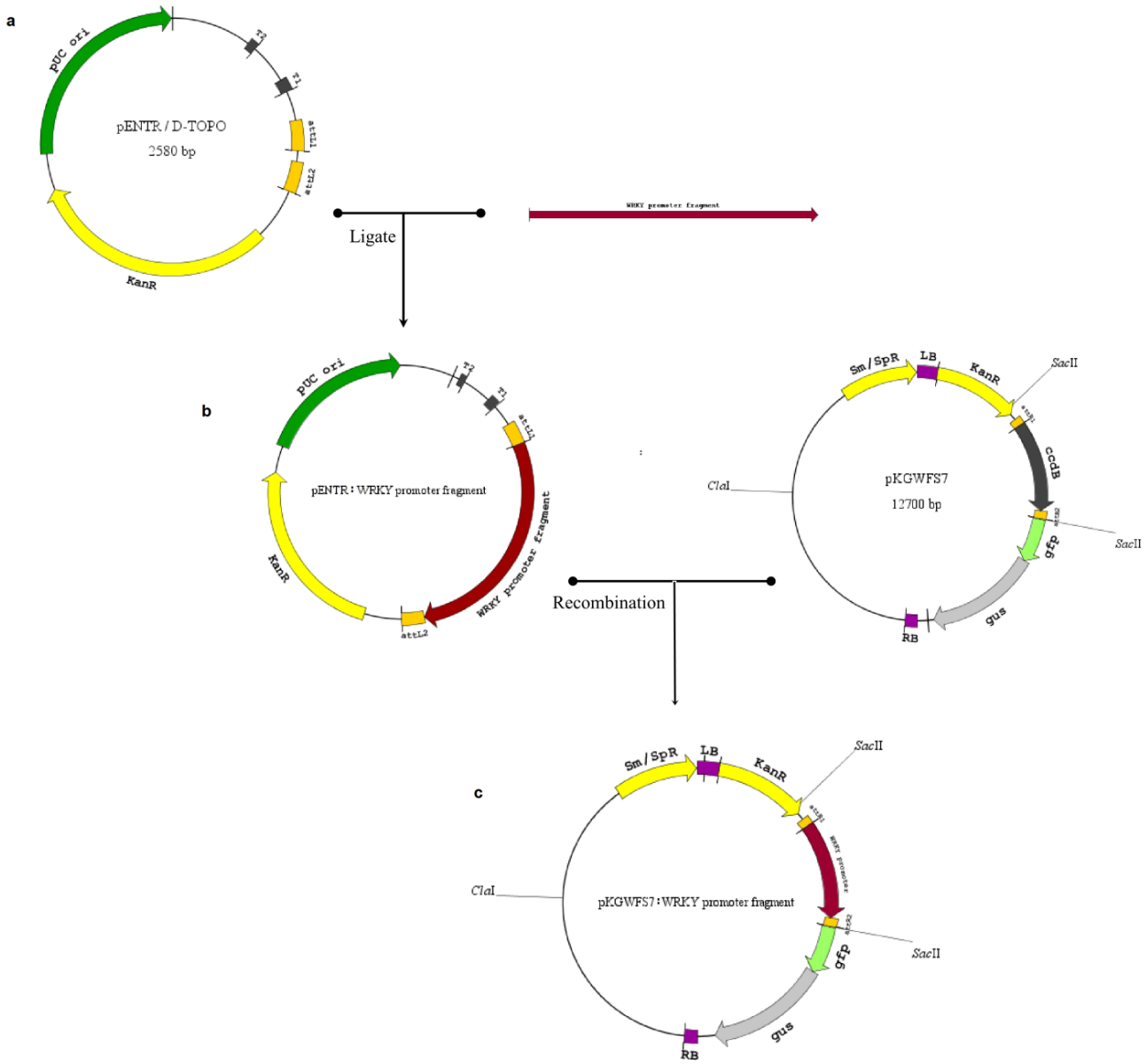

Fig.2 Construction of pKGWFS7:Glyma05g36970$_{500bp\ prom}$, pKGWFS7:Glyma05g36970$_{2000bp\ prom}$, pKGWFS7: Glyma08g15050$_{500bp\ prom}$, pKGWFS7: Glyma15g11680$_{500bp\ prom}$ and pKGWFS7: Glyma15g11680$_{2000bp\ prom}$ vectors by Gateway Cloning System method. (A) The 0,5 kbp and 2 kbp promoter fragments upstream the translation start site of Glyma05g36970 and Glyma15g11680 genes and the 0.5 kbp promoter fragment of Glyma08g15050 were introduced into the pENTR/D-TOPO cloning vector. (B) The formed entry vectors were recombined with the destination vector pKGWFS7, (C) thus forming the expression vectors.

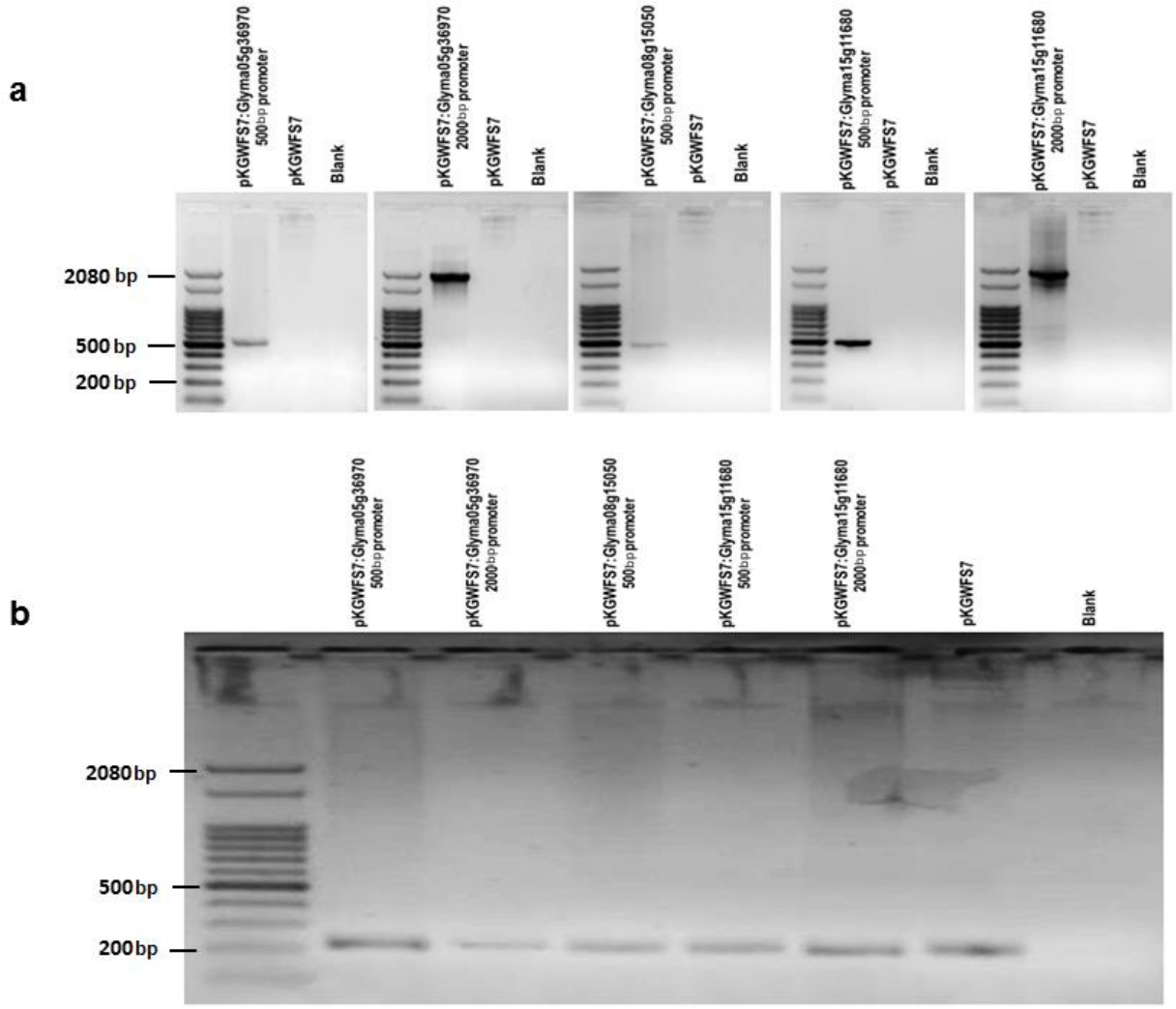

Fig. 3 Expression vectors obtained by the Gateway cloning method. 1% Agarose gel showing the following amplification products: (A) 2000pb/500pb promoter fragment upstream the translation start site of WRKY genes and (B) 228 bp fragment that identifies the presence of gusA gene.

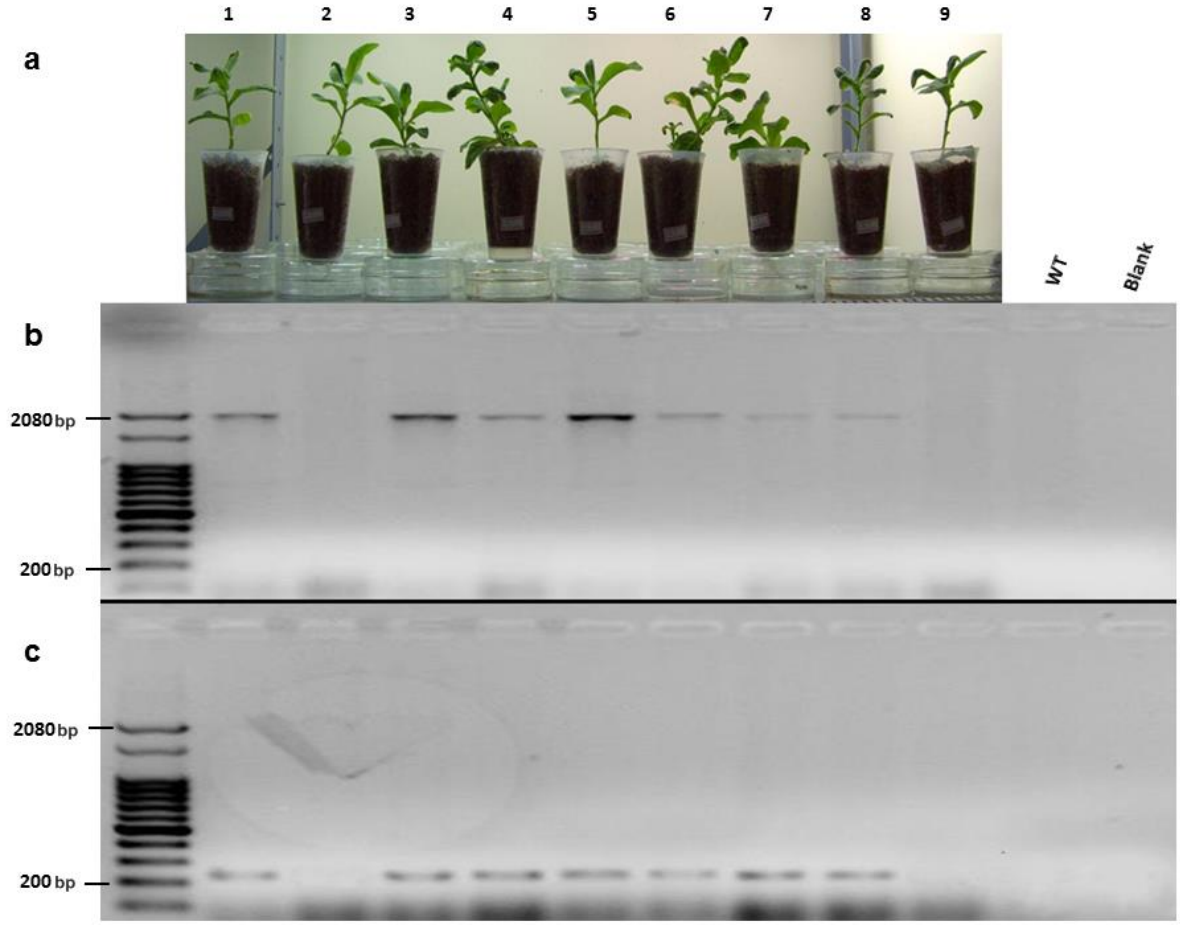

Fig. 4 PCR amplification of (B) 2 kb promoter fragment upstream the transcription start site of Glyma08g15050 gene and (C) 228pb fragment that identifies the presence of uidA gene (gusA) from (A) nine putative transgenic plants. Molecular weight marker is given on the left in base-pairs (bp). Lanes 1 to 9 correspond to regenerated tobacco plants. Negative controls: WT (Wild-Type tobacco) and Blank sample (distilled water).

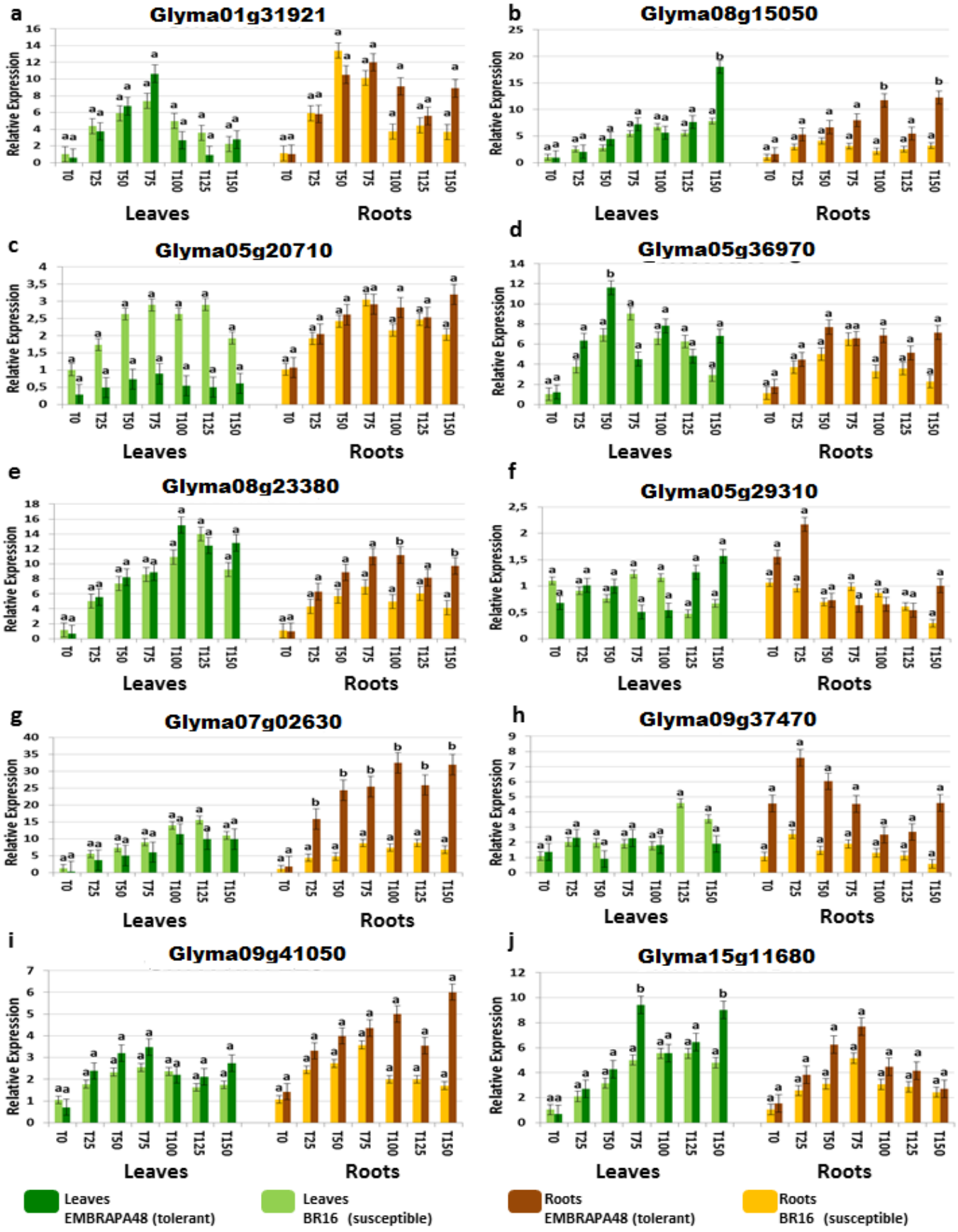

Fig. 5 Comparison of WRKY genes expression levels between cultivars within each organ (leaves / roots) in response to dehydration stress. The relative expression levels of genes were measured by RTqPCR at T0 (control), T25, T50, T75, T100, T125, and T150 minutes of dehydration stress. The values are the means of three biological replicates with four technical replicates each. The means that are labeled identically (with a letter) in the same organ do not differ significantly (Bonferroni multiple comparison test, $p<0.05$). The f-box, ACTII, and ELF reference genes were used as internal controls to normalize for the amount of mRNA present in each sample. The transcript levels from plants at T0 were used to normalize the transcript levels from plants subjected to dehydration stress.

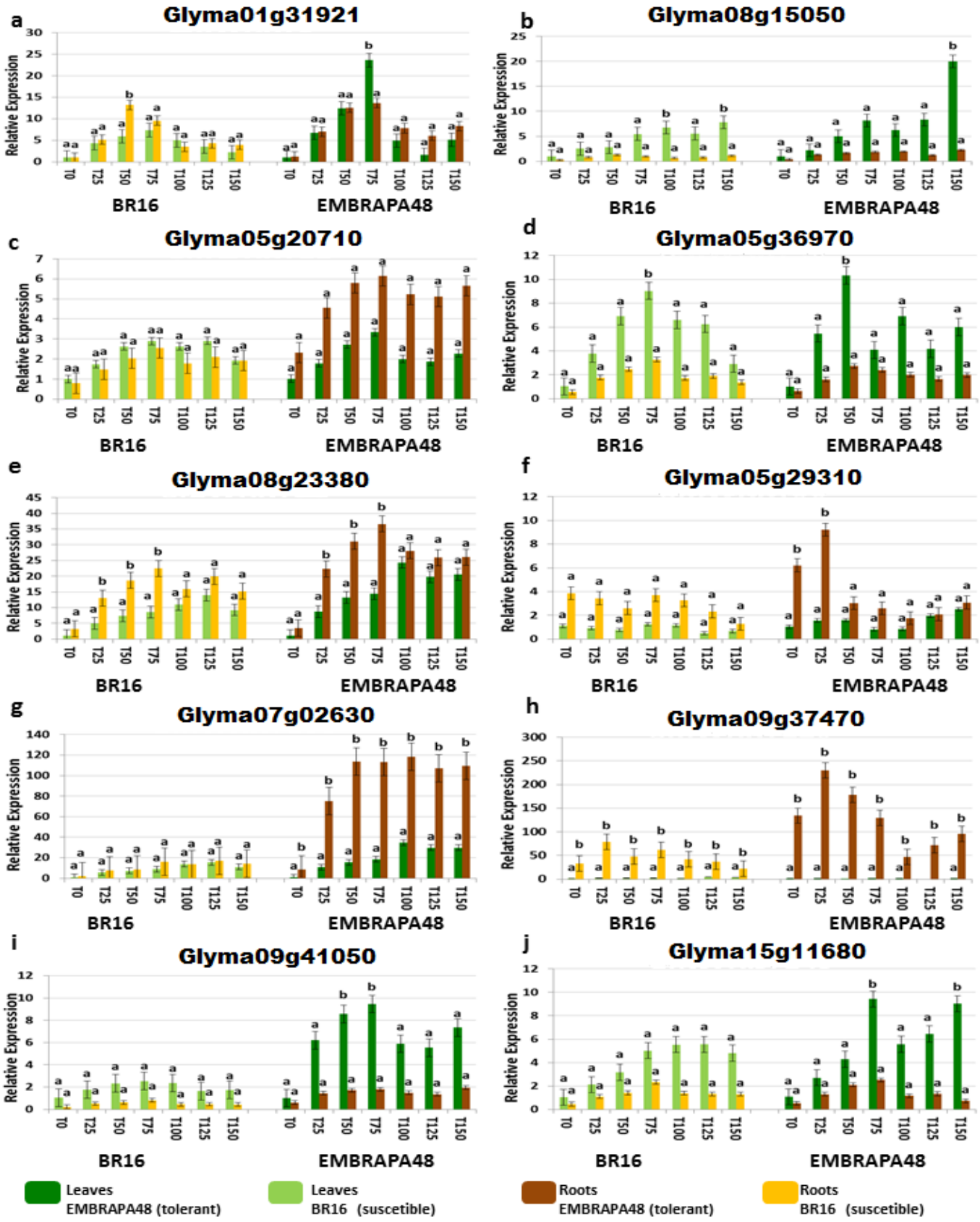

Fig. 6 Comparison of WRKY genes expression levels between organs (leaves x roots) within each cultivar in response to dehydration stress. The relative expression levels of the genes were measured by qPCR at T0 (control), T25, T50, T75, T100, T125, and T150 minutes of dehydration stress. The values are means of three biological replicates with four technical replicates each. The means that are labeled identically (with a letter) in the same cultivar do not differ significantly (Bonferroni multiple comparison test, $p<0.05$). The f-box, ACTII, and ELF reference genes were used as internal controls to normalize the amount of mRNA present in each sample. The transcript levels from plants at T0 were used to normalize the transcript levels from plants subjected to dehydration stress.

CONCLUSÕES E PERSPECTIVAS

4 CONCLUSÕES E PERSPECTIVAS

A trajetória do uso da engenharia genética para a obtenção de plantas tolerantes a estresses pode ser dividida em fases. A primeira tem como base a expressão de genes codificadores de proteínas funcionais e estruturais envolvidas na defesa vegetal. A partir da segunda fase o conceito de "inteligência vegetal" passou a ser considerado e estratégias moleculares mais eficazes na promoção e controle dos mecanismos de resistência das plantas foram elaboradas.

Apesar da ausência de um controle centralizador evidente, como um cérebro, a inteligência das plantas é entendida como um processo emergente do sistema. Ou seja, devido à integração e influência mútua entre milhões de células organizadas em algumas dezenas de tecidos, a planta percebe, processa e responde a informação transmitida pelo ambiente, exercendo sua plasticidade fenotípica (Souza 2009). Fundamentais na arquitetura dessa grande rede sinalizadora, os fatores de transcrição são os personagens principais da segunda fase. A superexpressão de genes rapidamente responsivos a estresses, como os codificantes de fatores de transcrição, acelera a ativação dos genes a jusante na cadeia de transdução do sinal e intensifica a resposta de defesa da planta (Zhu 2002).

Os perfis de transcrição determinados neste trabalho apontam os genes Glyma05g36970, Glyma07g02630, Glyma08g15050, Glyma08g23380 e Glyma15g11680 como potenciais candidatos para aplicação em transformação genética de plantas visando incremento da tolerância ao déficit hídrico. A expressão diferencial destes genes entre as cultivares de soja suscetível e tolerante à seca sugere o envolvimento dos mesmos nas rotas que compõem a defesa da soja à desidratação. Além disso, a possibilidade do uso desses genes como ferramentas de fenotipagem em programas de melhoramento também deve ser considerada. Testes que de forma rápida e direta permitam avaliar o comportamento dos genótipos em condições de estresse são de grande valor para guiar o melhorista na tomada de decisões. Por isso, a quantificação do nível de expressão de sinalizadores-chave e/ou fatores de

transcrição que são consistentemente induzidos ou reprimidos em resposta ao estresse é um método interessante de fenotipagem (Setter 2012).

A atual fase da engenharia genética aposta nos promotores sintéticos para o desenvolvimento de cultivares tolerantes a estresses. Os promotores sintéticos contêm as mesmas sequências de DNA (*cis*-elementos) dos promotores nativos de plantas, porém estas sequências são organizadas e condensadas de uma forma não encontrada na natureza (Liu e col. 2013). Os promotores gênicos são as unidades de regulação transcricional da expressão gênica, sendo assim, a oportunidade de "construí-los" torna mais factível o alcance de um controle preciso da expressão dos transgenes. Entretanto, a construção de promotores sintéticos ainda é limitada pela necessidade de um conhecimento mais profundo sobre o papel dos elementos cis regulatórios e a influência que o arranjo dessas sequências tem sobre a variabilidade de suas funções.

No presente trabalho, fragmentos de 500bp e 2000bp a montante do sítio de início da transcrição dos genes Glyma05g36970, Glyma08g15050 e Glyma15g11680 foram fusionados ao gene repórter e clonados em vetores adequados para transformação genética de plantas (Fig.1 e 2). Esses vetores, por sua vez, foram introduzidos em plantas de tabaco via *Agrobacterium tumefaciens*, resultando na obtenção de sete plantas transgênicas que carregam a construção "2000bp promotor Glyma08g15050: gusA" (Fig. 4). Os transformantes referentes às demais construções estão em processo de seleção. As plantas transgênicas obtidas serão empregadas em ensaios para avaliação da atividade dos promotores. Características como facilidade de manipulação *in vitro*, boa taxa de crescimento e grande produção de biomassa (folhas grandes) fizeram do tabaco uma planta modelo em experimentos de transformação genética.

Embora os sistemas heterólogos sejam legítimos e bastante utilizados para análise de promotores, experimentos de transformação serão realizados em soja. Devido às muitas diferenças entre as espécies, como anatomia dos tecidos e padrões de expressão, que podem ocasionar alterações na expressão dos construtos, a validação da atividade das sequências promotoras

deve também ser realizada no organismo nativo (Hernandez-Garcia e col. 2010).

O potencial dos promotores em estudo na resposta à desidratação também será avaliado. Para isso as sementes das plantas transgênicas de tabaco e, a médio prazo, de soja obtidas serão germinadas e as plântulas produzidas serão submetidas ao déficit hídrico por meio da adição de polietilenoglicol (PEG) ao meio de cultura. O PEG é um polímero utilizado para modificar o potencial osmótico do meio induzindo o déficit hídrico nas plantas, de uma forma relativamente controlada, adequada para protocolos de testes experimentais iniciais. A investigação do comportamento destes promotores sob a ação de outros tipos de estresses e fitohormônios também é interessante tanto para elucidar a participação dos genes por eles dirigidos em outras vias de resposta da planta como também para compreender a atribuição de cada cis-elemento para a construção destas respostas.

REFERÊNCIAS DOS TÓPICOS “INTRODUÇÃO GERAL”

E “CONCLUSÕES PERSPECTIVAS”

5 REFERÊNCIAS DOS TÓPICOS "INTRODUÇÃO GERAL" E "CONCLUSÕES E PERSPECTIVAS"

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